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<p>(21) International Application Number: PCT/GB90/01131 (22) International Filing Date: 23 July 1990 (23.07.90) (30) Priority data: 8916806.6 22 July 1989 (22.07.89) GB (71) Applicant (for all designated States except US): UNIVERSITY OF WALES COLLEGE OF MEDICINE [GB/GB]; Heath Park, Cardiff CF4 4XN (GB). (72) Inventor; and (75) Inventor/Applicant (for US only): CAMPBELL, Anthony, Keith [GB/GB]; 14 Maillard's Haven, Penarth, S. Glamorgan CF6 2RF (GB). (74) Agents: NEWELL, William, Joseph et al.; Wynne-Jones, Laine & James. Morgan Arcade Chambers, 33 St. Mary Street, Cardiff CF1 2AB (GB).</p>	<p>(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CM (OAPI patent), DE*, DE (European patent)*, DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US. Published With international search report.</p>	
<p>(54) Title: MODIFIED BIOLUMINESCENT PROTEINS AND THEIR USE</p> <p>(57) Abstract</p> <p>A modified bioluminescent protein responds to different physical, chemical, biochemical or biological conditions to produce light or radiation of altered characteristics when the bioluminescent reaction is initiated. The modified bioluminescent protein may respond to modification thereof, e.g. by covalent modification. The protein may include signal peptides to "target" it. DNA coding for the bioluminescent protein may be altered to include tissue specific promoter or enhancer genes so that the altered DNA acts as reporter gene.</p>		

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Modified bioluminescent proteins and their use.

This invention relates to bioluminescent proteins, in particular it relates to bioluminescent proteins which have been modified, for example by chemical means or by genetic engineering. Such modified bioluminescent proteins, hereinafter referred to as "rainbow proteins", may be used in the detection and quantification of cells, microbes such as bacteria, viruses and protozoa, and substances of biological interest such as substrates, metabolites, intra- and extra- cellular signals, enzymes, antigens, antibodies and nucleic acids.

Bioluminescence is the oxidation of an organic molecule, the "luciferin", by oxygen or one of its metabolites, to emit light. The reaction is catalysed by a protein, usually known as a "luciferase"; or a "photoprotein" when the luciferin is so tightly or covalently bound to the luciferase that it does not diffuse off into the surrounding fluid.

$$O_2 + \text{luciferin} + \text{luciferase} \rightarrow \text{oxyluciferin} + \text{light}$$

(or O_2^- or H_2O_2) (or photoprotein)

Up to three other substances may also be required to be present in order to generate light, or to alter its colour, and they are as follows:-

(a) A cation such as H^+ , Ca^{2+} , Mg^{2+} , or a transition

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metal such as $\text{Cu}^+/\text{Cu}^{2+}$, $\text{Fe}^{2+}/\text{Fe}^{3+}$.

(b) A cofactor such as NADH, FMN, or ATP.

(c) A fluor as an energy transfer acceptor.

Five chemical families of luciferin have been identified so far (see Figure 1 of the attached drawing):

(a) Aldehydes (found in bacteria, freshwater limpet Latia and earthworms).

(b) Imidazolopyrazines (found in Sarcomastigophora, Cnidaria, Ctenophora, some Arthropoda, some Mollusca, some Chordata).

(c) Benzothiazole (found in beetles such as fireflies and glowworms).

(d) Linear tetrapyrroles (found in dinoflagellates, euphasiid shrimp, some fish).

(e) Flavins (found in bacteria, fungi, polychaete worms and some molluscs).

Reactions involving these luciferins may result in the emission of violet, blue, blue-green, green, yellow or red light and occasionally UV or IR light- and such emission may or may not be linearly or circularly polarised. Reference is directed to Chemiluminescence principles and applications in biology and medicine, A.K. Campbell, publ. 1988 Horwood/VCH Chichester Weinheim, for further discussion of bioluminescent reactions.

It has now been found that the light emitted from a

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bioluminescent reaction involving a modified bioluminescent or "rainbow" protein, may be changed in intensity, colour or polarisation. Such a change can then be used in various assays for detecting, locating and measuring cells, microbes and biological molecules.

In this instance, the cell or substance causes a physical or chemical change, such as phosphorylation, to a rainbow protein such as a genetically engineered luciferase, resulting in a change in intensity, colour or polarisation of the light emission. The bioluminescent reaction is triggered by adding, for example, the luciferin, and modification of the luciferase by the cell or substance being measured causes the reaction to emit light at a shorter or longer wavelength. This enables specific reactions to be detected and quantified in live cells, and within organelles or on the inner or outer surface of the plasma membrane, without the need to break them open, and without the need for separation of bound and unbound fractions.

According to one aspect of the invention there is provided a bioluminescent protein capable of taking part in a bioluminescent reaction to produce light or radiation of altered characteristics under different physical, chemical, biochemical or biological conditions.

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The rainbow protein may be produced by the alteration, substitution, addition or removal of one or more amino acids from the end of or within the luciferase or photoprotein. As a result the light emission from the oxyluciferin may be of different colours or different states of polarisation depending on the physical or chemical conditions. A change in colour to another part of the rainbow spectrum may be induced by:

- (a) A change in cation concentration such as H⁺, Ca²⁺, Mg²⁺, or transition metal.
- (b) A change in anion concentration such as Cl⁻ or phosphate.
- (c) Covalent modification of the new protein by enzymes causing phospho- or dephosphorylation (including ser/thr, his, and tyr kinases and phosphatases) transglutamination, proteolysis, ADP ribosylation, gly- or glu-cosylation, halogenation, oxidation, methylation and myristilation.
- (d) Binding to the rainbow protein of an antigen, an intracellular signal such as cyclic AMP, cyclic GMP, Ip3, Ip4, diacyl glycerol, ATP, ADP, AMP, GTP, any oxy or deoxyribonucleoside or nucleotide, a substrate, a drug, a nucleic acid, a gene regulator protein.
- (e) Expression of its nucleic acid inside a live cell, as well as its modification^{or regulation} within the cell by

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gene expression such as promoters, enhancers or oncogenes.

Single or multiple mutations and deletions may be detected in a piece of DNA (eg a PCR product) by linking the "rainbow protein" to one end of the DNA and an energy transfer acceptor or quencher to the other end. Nuclease attack at the mutation will separate the rainbow protein from the acceptor or quencher and thus cause a change in intensity, colour or polarisation of the light emission.

Such alteration, substitution, addition or removal of one or more amino acids may be achieved by chemical means. Alteration of an acid includes alkylation (eg. methylation), phosphorylation and various other covalent modifications of the type outlined herein. Alternatively the nucleic acid coding for the luciferase or photoprotein may be altered by modifying, substituting, inserting or deleting one or more nucleotides such that the resulting protein has gained or lost a site which interacts with the cations, anions, intracellular signal, covalent modification; proteins or nucleic acid to be measured. The insertion or deletion of nucleotides is normally produced by site directed mutagenesis or by opening up the gene with a specific restriction enzyme, inserting or deleting a selected nucleotide sequence and then sealing up of the gene again or using specific primers with the polymerase

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chain reaction. The nucleic acid is then transcribed to mRNA and this is then translated to form the rainbow protein either inside a bacterial or eukaryotic cell, or in vitro using, for example, rabbit reticulocyte lysate. The new nucleic acid may contain an RNA polymerase promoter such as T7, SP6, or mammalian promoters such as actin, myosin, myelin proteins, MMT-V, SV40, antibody, G6P dehydrogenase, and can be amplified in vitro using the polymerase chain reaction. The result is that the rainbow protein can be produced either in a live cell such as a cancer cell, or without cells using enzymatic reactions in vitro. The addition of tissue specific promoter or enhancer sequences to the 5' or 3' end of the DNA coding for the native or altered bioluminescent protein will enable it to be used as a reporter gene and to be expressed specifically in a particular cell or tissue, the expression being detectable by the appearance of a change in light intensity, colour or polarisation.

Another way of producing the DNA for a rainbow protein is to separate into two halves the original DNA for the bioluminescent protein. A piece of DNA or gene is then inserted between the two halves by ligating one half to the 5' end and the other to the 3' end. Alternatively the rainbow protein DNA could be generated using the polymerase chain reaction so that the sense

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primer had one part of the rainbow protein DNA linked at 5' end and the antisense primer and the other part linked at the 3' end (i.e. antisense). The pieces of DNA or gene of interest, in the middle, could be from two separate genes. For example one could code for an energy transfer protein, the other for a bioluminescent protein. Only when the two are linked together via a peptide (from DNA/RNA) will the rainbow protein be generated and a shift in colour occur. The energy transfer protein could be any fluor bound covalently or non-covalently to the protein, for example the green fluorescent protein from *Aequorea*, *Obelia*, *Renilla* or other cnidarians, or the blue or yellow fluorescent protein from luminous bacteria, or a flavoprotein, or a phyobiloprotein. The whole protein or just the fluorescent domain may be used. The bioluminescent protein would be any luciferase for example bacterial, firefly, glowworm or copepod, or any photoprotein for example aequorin, obelin or a radiolaxin such as thalassicollin.

The protein or its DNA or RNA may be incorporated into a live bacterium or eukaryotic cell by using a virus, plasmid, calcium phosphate transfection, electroporation, liposome fusion or membrane pore forming proteins. Once inside, only live cells with the appropriate biochemistry will produce the "rainbow

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effect". By incorporating the "rainbow protein" gene into an embryo, oocyte, sperm, seed or seedling a transgenic animal or plant may be produced, enabling gene expression, cell regulation, drug action, or cell damage to be located and measured in individual organs using the "rainbow effect". These new organisms may also be used in home aquaria, on aeroplane runways, as safe lights at sea, and as house plants.

The rainbow protein may also be incorporated in a different part of the cell by chemical means or genetically engineering the protein to contain a signal peptide which locates it to the inner or outer surface of the plasma membrane or within a particular intracellular organelle (e.g. peroxisome, mitochondrion, chloroplast, endoplasmic reticulum, golgi, secretory vesicle, nucleus or endosome).

Addition of a signal peptide, either chemically or by genetic engineering, will enable the normal or altered luciferase or photoprotein to be targetted into a specific organelle within the cell, or onto the inner or outer surface of the plasma membrane. For example the sequence MLSRLSLRLLSRYLL at the N terminus will locate the bioluminescent protein in the mitochondria, and KKSALLALNIYVCPGKADKE on the N terminus will target the protein to the endoplasmic reticulum, a KDEL sequence at the C terminus retaining it there.

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The initial luciferase or photoprotein or its gene may come from any of the known chemistries in bioluminescence (see Figure 1) or from the wide range of uncharacterised luminous organisms from more than 700 genera representing at least 16 phyla. The luciferin may be synthesised chemically and added to the biological reaction or cell to generate light. Alternatively, the gene coding for the enzymes responsible for making the luciferin may be linked to the "rainbow protein" gene so that the artificial operon or fusion gene expresses both rainbow protein and makes luciferin in the live cell from normal cell constituents such as amino acids.

According to a second aspect of the invention there is provided a method of producing a bioluminescent protein by altering, substituting, adding or deleting one or more amino acids to the protein by chemical means or by genetically engineering the nucleic acid coding for the protein.

According to a further aspect of the invention there is provided nucleic acid coding for the bioluminescent protein as hereinbefore defined.

The rainbow protein, or the nucleic acid coding for it, may be used in a range of biological investigations; such as:-

(a) Detection, location and measurement of microbes

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(protozoa, bacteria, viruses).

(b) Detection and location of cancer cells.

(c) Measurement of enzymes, intracellular signalling and other turnover reactions in cells or fluids.

(d) DNA and RNA binding assays.

(e) Immunoassay and other protein binding assays.

The rainbow proteins and their parent nucleic acids also may be used in genetic engineering, in the development of transgenic animals, plants and microbes, and in horticulture.

According to yet a further aspect of the invention there is provided the use of a rainbow protein, or the nucleic acid coding for the rainbow protein, for the detection, location or measurement of substances of biological interest such as microbes, cells or biological molecules or reactions therein.

In this aspect, the reaction or substances of biological interest are made to interact with the rainbow protein or its parent nucleic acid. Such interactions include direct or indirect linking such as non-covalent or covalent binding as well as energy transfer processes.

Although the invention has been described above it is to be understood that it includes any inventive combination of the features set out above or in the following description.

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The invention may be performed in various ways and will be further described with reference to the following examples:

EXAMPLE 1

Detection of phosphorvlation of a rainbow protein

The peptide leu arg arg ala ser leu gly, known as kemptide, or RTKRSCSVYEPLKT known as malantide was covalently linked to firefly luciferase using disuccinyl suberate at pH8. Addition of 125 ul protein kinase A + cyclic AMP (200 uM) + 125 uM ATP caused phosphorylation of the kemptide, now attached to the luciferase. The resulting shift in colour from yellow-green to red at pH 7.8 or from red to yellow-green at pH 6.5, measured as a ratio in a dual wavelength chemiluminometer, enabled the rate of protein phosphorylation by the kinase to be assayed, and dephosphorylation induced by phosphatase to be assayed subsequently by the reversal of the ratio.

EXAMPLE 2Engineering Firefly Luciferase

cDNA coding for firefly luciferase was amplified using the polymerase chain reaction (PCR) using 5' sense primers with a T7 RNA polymerase promoter, and 3' antisense primers as follows: Primer code in brackets

5' sense primers

- (105) CACCTAATACGACTCACTATAGGGAGAATGGAAGACGCCAAAAAC
(107) AGAACTGCCTGCCGCAGATTCTCGCA
(110) ATGCTGTCCCGGCTGTCCCTGCGGCTGCTGTCCCGGTACCTGCTGAAGACGC
CAAAAAAC
(111) CACCTAATACGACTCACTATAGGGAGAATGCTGTCCCGGCTGTCC

3' antisense primers

- (100) TCTCGCTGAATACAGTTAC
(106) CCCCAAGCTTAGATCTCTCTGATTTTCTTGCGT
(108) TGCGAGAATCTGCGGCAGGCAGTTCT

The following firefly luciferase cDNA's were constructed using primers in brackets:

- (a) full length (105 + 100)
(b) - 36bp i.e. missing peroxisomal signal peptide (105 + 106)
(c) protein kinase A site (RRXS) in middle of protein
(step 1: 105 + 108 and 107 + 100; step 2: 2 halves from
step 1 + 105 + 100)
(d) mitochondrial signal at N terminus (step 1: 110 + 100;
step 2: step 1 sample + 111 + 100).

The PCR reaction in 50 μ l contained 10 mM Tris pH 8.3, 0.01% gelatin, 0.4U Taq polymerase, 2 mM $MgCl_2$, 0.2 mM each dATP, dGTP, dTTP, dCTP, 0.5 μ M of each primer, 1 μ l DNA (ca 1-100 ng). The reaction, covered with 50 μ l mineral oil, was incubated in a Perkin-Elmer thermal cycler for 25 cycles: 94°C 1 minute, 55°C 1 minute, 72°C 2 minutes + 5 seconds extension on each cycle, then for 30 minutes at 37°C with 1U E.coli DNA polymerase (Klenow fragment).

Successful PCR was confirmed by a band on agarose gel electrophoresis. The cDNA was purified by centricon to remove primers, and precipitated in 70% ethanol, 0.7 mM NH_4 acetate after extraction with buffered phenol: $CHCl_3$: secondary amyl alcohol (25:24:1). The DNA (0.5-1 μ g dissolved in 10mM Tris 0.1 or 1 mM EDTA pH 7.4-7.5) was transcribed in the T7 RNA polymerase in buffer containing 40 mM Tris, pH 7.4-7.5, 6mM $MgCl_2$, 10 mM dithiothreitol, 0.1mg/ml bovine serum albumin, 2 mM spermidine, 0.5 mM each ATP, CTP, UTP, 0.1 mM GTP, 0.5 mM cap m7 G(5') ppp (5') G, 1000 U RNAsin/ml, 800 U T7 RNA polymerase \pm 2 μ C, ^{32}P UTP for up to 4 hours (1-2 hours optimal), at 37°C. The reaction was stopped in the ice cold phenol: $CHCl_3$: secondary amyl alcohol (25:24:1), and the RNA precipitated in 70% ethanol + 0.7M NH_4 Ac, and stored at -20°C.

The RNA was centrifuged, redissolved in 20 μ l 70 mM Tris, 1 mM EDTA pH 7.4-7.5 and 1 μ l incubated with 5-10 μ l rabbit reticulocyte lysate for 1 hour at 30°C to synthesize the luciferase. Luciferase, after dilution, 1/100 is assayed for light emission directly in 50 mM tris, 10 mM $MgAc_2$, 0.1

mg/ml bovine serum albumin, 0.1-0.2 mM luciferin, 0.5-5 mM ATP, pH 7.8, or isolated by isoelectric focusing. The mutant (RRXS) luciferase has a pI of ca 7.1 or 6.8, and the normal luciferase pI ca 6.8. The luciferase with mitochondrial signal also separated from the normal luciferase. On addition of the rabbit reticulocyte lysate containing this altered luciferase it was taken up by added mitochondria, as shown by centrifugation and light emission from the mitochondria and luciferase.

Phosphorylation of the RRXS containing luciferase with protein kinase A, cyclic AMP (0.2 mM), ATP (0.1 - 1 mM) pH7, caused the luciferase to change its pI back towards 6.8, and to shift its colour. The RRXS luciferase had a greener light emission than the native luciferase detected using a dual wavelength chemiluminometer with interference filters (maximum transmission ca 545 and 603 nm).

Primers containing kemptide nucleotide sense or antisense sequence (LRRASLG) or malantide RTKRSGSVYEPLKI were also added either to N or C terminus using a one or two step PCR reaction. These also produced luciferase which could be phosphorylated thereby altering its intensity and colour.

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EXAMPLE 3Preparation of engineered aequorin

cDNA or genomic coding for the Ca^{2+} -activated photoprotein was PCR'd in a similar way to that for firefly luciferase. Using one or two step PCR the protein kinase A recognition peptide kemptide (LRRLALG) or malantide (as Example 2) was added to the N terminus. The mutant aequorin had different kinetic properties enabling protein kinase A to be detected by phosphorylating the altered aequorin (above).

Normal aequorin primers =5' sense TAATACGACTCA CTATAGGGGAGAGAATGGTCAAGCTTTACATCAGACTTCGAC, and 3' antisense GAATTCTTAGGGGACAGCTCCACCGTA. For insertion of kemptide at the N terminus the nucleotide sequence equivalent to LRRASLG was attached to the first 15 bases (including ATG) of the 5' end of aequorin. In step 2, the T7 RNA polymerase promoter was added to form the kemptide-aequorin in vitro for in vitro phosphorylation. Genomic aequorin DNA (made by PCR) was at least as active as that made from mRNA by reverse transcriptase PCR.

EXAMPLE 4Detection of cancer cells in blood

A blood sample (1 ml) is mixed with a suspension of liposomes containing mRNA coding for a rainbow protein catalysing the benzothiazole reaction c in Figure 1.

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This mRNA was produced as follows:-

The gene coding for firefly luciferase was first isolated from a cDNA library in *E. coli* using pCDV1 plasmid primer + Honjo linker containing SP6 RNA polymerase promoter. A nucleotide sequence GCTCGTCTTATTGAAGATAATGAATATACTGCTCGTTTTGGT representing the phosphorylation site for tyrosine kinase activity of the myc oncogene was inserted at the EcoRI restriction site 30 base pairs downstream from the ATG at the 5' end. The DNA was resealed, recloned and the plasmid insert transcribed by SP6 RNA polymerase in vitro to produce mRNA for the rainbow protein. The original protein produced yellow-green light but the rainbow protein when phosphorylated in the cell produces red light. Thus the presence of cancer cells was detected in blood sample from a leukaemia patient by measuring the ratio of yellow-green (545 nm) to red (603 nm) light in a dual wavelength chemiluminometer.

EXAMPLE 5

Detection of Salmonella

The cDNA for the rainbow protein in Example 4 containing SP6 RNA polymerase promoter was inserted into Salmonella phage. Addition of this phage to Salmonella resulted in expression of the rainbow protein and the generation of red light, enabling as few as 1 bacterium

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per 20 ml to be detected.

EXAMPLE 6

Detection of HIV RNA

A sample (1 ml) of blood from a patient with AIDS was extracted with 4 M guanidium isothiocyanate and the nucleic acid precipitated with ethanol/NH₄ acetate. An oligonucleotide (10 ul, 1 uM) labelled with a rainbow protein generated from the photoprotein obelin was added to 100 ul redissolved RNA at 50°C and the mixture cooled for 10 minutes at 0°C. The oligonucleotide was specific for a sequence in the HIV coat protein. Binding this to HIV RNA resulted in a shift in the light emission from the rainbow protein from light blue (475 nm) to blue (440 nm). This was detected as a shift in the ratio of light emission at these two wavelengths in a dual photomultiplier chemiluminometer.

EXAMPLE 7

Measurement of testosterone in blood

Testosterone carboxyoxime is reacted with the rainbow protein from the photoprotein obelin to form a testosterone rainbow protein conjugate. 5ul of this containing 1 nmol was incubated with a solution of antibody labelled with fluorescein to testosterone (50ul) pH 7.4 for 30 minutes in the presence or absence of

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present the ratio of ratios is 1.

EXAMPLE 9

Measurement of nucleic acid hybridisation by polarisation

The reaction described in Example 6 was carried out but the light emission was detected in a dual photomultiplier chemiluminometer containing two plane polarised filters with the polarisation planes at 90° to each other. The ratio between the two photomultipliers was related to the amount of HIV RNA present.

EXAMPLE 10

Measurement of cyclic AMP or Ip3

Using a two step PCR reaction as described in Examples 2 and 3, the cyclic AMP binding domain from the bacterial CAP protein or the Ip3 binding domain of the endoplasmic reticulum receptor was added to the N or C terminus or into firefly luciferase or aequorin. The altered proteins were made in vitro from the PCR DNA product as described in Examples 2 and 3, and characterised by activity and colour of light emission cyclic AMP or Ip3. A change in both intensity and colour enabled CAMP or Ip3 to be measured in cell extracts or in living cells. Using an image intensifier a CAMP or Ip3 "cloud" could be visualised in this one

CLAIMS

1. A bioluminescent protein capable of taking part in a bioluminescent reaction to produce light or radiation of altered characteristics under different physical, chemical,
5 biochemical or biological conditions.

2. A bioluminescent protein according to Claim 1, wherein said altered characteristics include one or more of colour, polarization state, intensity.

3. A bioluminescent protein according to Claim 1 or 2,
10 produced by modification of a bioluminescent protein precursor.

4. A bioluminescent protein according to Claim 3, wherein said modification includes the alteration, substitution, addition or removal of one or more amino acids at the
15 N or C terminus or within the protein.

5. A bioluminescent protein according to any preceding claim, responsive to binding or association of the protein with a substance of interest to produce light or radiation of altered characteristics when said bioluminescent reaction
20 is initiated.

6. A bioluminescent protein according to any of Claims 1 to 4, responsive to covalent or other modification of the protein to produce light or radiation of altered characteristics when said bioluminescent reaction is initiated.

25 7. A bioluminescent protein according to any of Claims

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reporter gene.

14. A nucleic acid according to Claim 12 or 14,
including a sequence coding for one or more substances for
producing luciferin or a similar molecule capable of
5 emitting light or radiation.

15. The use of a bioluminescent protein according to
any of Claims 1 to 10 for the detection, location, measure-
ment or visualization of substances within or outside
microbes, whole tissues, whole organisms, cells or
10 biological molecules.

16. A method of detecting mutations or detections in
a DNA sequence includes linking a bioluminescent protein
according to any of Claims 1 to 10 to one end of the
sequence and an energy transfer acceptor or quencher to the
15 other end.

INTERNATIONAL SEARCH REPORT

PCT/GS 90/01131

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ¹ According to International Patent Classification (IPC) or to both National Classification and IPC: IPC ⁵ : C 07 D 13/00, C 07 K 15/08, C 12 N 15/12, C 12 Q 1/66, C 12 O 1/68, G 01 N 33/542													
II. FIELDS SEARCHED <div style="text-align: right; font-size: small;">Minimum Documentation Searched⁷</div> <div style="text-align: right; font-size: small;">Classification Symbols</div> Classification System: IPC ⁵ C 07 K, C 12 N, C 12 Q, G 01 N													
<div style="text-align: center; font-size: x-small;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched⁸</div>													
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="text-align: left; font-size: x-small;">Category¹⁰</th> <th style="text-align: left; font-size: x-small;">Citation of Document, ¹¹ with Indication, where appropriate, of the relevant passages¹²</th> <th style="text-align: left; font-size: x-small;">Relevant to Claim No.¹³</th> </tr> <tr> <td style="vertical-align: top; padding: 5px;">X</td> <td style="vertical-align: top; padding: 5px;">Proc. Natl. Acad. Sci. USA, volume 83, no. 21, 3-14 November 1986 F.I. Tsuji et al.: "Site-specific mutagenesis of the calcium-binding photoprotein aequorin", pages 8107-8111 see the whole article --</td> <td></td> </tr> <tr> <td style="vertical-align: top; padding: 5px;">X</td> <td style="vertical-align: top; padding: 5px;">Proc. Natl. Acad. Sci. USA, volume 86, no. 1, 3-14 January 1989 K. Kurose et al.: "Bioluminescence of the Ca²⁺-binding photoprotein aequorin after cysteine modification" see the whole article --</td> <td></td> </tr> <tr> <td style="vertical-align: top; padding: 5px;">X</td> <td style="vertical-align: top; padding: 5px;">Chemical Abstracts, volume 104, no. 17, 28 April 1986, (Columbus, Ohio, US), S.C. Alter et al.: "The sulfhydryls of firefly luciferase are not essential for activity" see pages 301-302, abstract no. 144584g. & Biochemistry 1986, 25(7) 1599-605 --</td> <td style="vertical-align: top; text-align: center; padding: 5px;">3-14</td> </tr> </table>		Category ¹⁰	Citation of Document, ¹¹ with Indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	X	Proc. Natl. Acad. Sci. USA, volume 83, no. 21, 3-14 November 1986 F.I. Tsuji et al.: "Site-specific mutagenesis of the calcium-binding photoprotein aequorin", pages 8107-8111 see the whole article --		X	Proc. Natl. Acad. Sci. USA, volume 86, no. 1, 3-14 January 1989 K. Kurose et al.: "Bioluminescence of the Ca ²⁺ -binding photoprotein aequorin after cysteine modification" see the whole article --		X	Chemical Abstracts, volume 104, no. 17, 28 April 1986, (Columbus, Ohio, US), S.C. Alter et al.: "The sulfhydryls of firefly luciferase are not essential for activity" see pages 301-302, abstract no. 144584g. & Biochemistry 1986, 25(7) 1599-605 --	3-14
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X	Proc. Natl. Acad. Sci. USA, volume 86, no. 1, 3-14 January 1989 K. Kurose et al.: "Bioluminescence of the Ca ²⁺ -binding photoprotein aequorin after cysteine modification" see the whole article --												
X	Chemical Abstracts, volume 104, no. 17, 28 April 1986, (Columbus, Ohio, US), S.C. Alter et al.: "The sulfhydryls of firefly luciferase are not essential for activity" see pages 301-302, abstract no. 144584g. & Biochemistry 1986, 25(7) 1599-605 --	3-14											
<div style="font-size: x-small;"> ¹⁰ Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed </div>													
<div style="font-size: x-small;"> ¹¹ "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention ¹² "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step ¹³ "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such docu- ments, such combination being obvious to a person skilled in the art "G" document member of the same patent family </div>													
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; vertical-align: top; padding: 5px;"> Date of the Actual Completion of the International Search 28th October 1990 </td> <td style="width: 50%; vertical-align: top; padding: 5px;"> Date of Mailing of this International Search Report 14 NOV 1990 </td> </tr> <tr> <td style="vertical-align: top; padding: 5px;"> International Searching Authority EUROPEAN PATENT OFFICE </td> <td style="vertical-align: top; padding: 5px;"> Signature of Authorized Officer Mme N. KUIPER </td> </tr> </table>		Date of the Actual Completion of the International Search 28th October 1990	Date of Mailing of this International Search Report 14 NOV 1990	International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer Mme N. KUIPER								
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FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claim numbers 1, 2, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The wording of claims 1 and 2 is not sufficient to characterise the subject matter for which protection is sought (see Art. 6 and Rule 6 PCT)

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.